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Studies on the Histogenesis of Blood Platelets and Megakaryocytes

Histochemical and Gasometric Investigations of Acetylcholinesterase Activity in the Erythrocyte-Erythropoietic and Platelet-Megakaryocytic Systems of Various Mammals

By

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I. Introduction.

Speculation concerning the origin of the blood platelets has produced an extensive literature. Almost every possible cell in the blood and in the blood-forming tissues has been suggested as the mother cell of the platelets. The literature was reviewed in detail by HITTMAIR (1928, 1938), ROSENTHAL (1938), TOCANTINS (1938), SALTZMAN (1948), and KISSMEYER-NIELSEN (1954).

Only two theories have attracted general attention. One, proposed by WRIGHT (1906), suggests that the platelets arise by budding from the cytoplasm of the megakaryocytes. The other, most energetically defended by SCHILLING (1950), maintains that the platelets originate from the nucleus of the normoblast. On the basis of studies which were largely clinical, morphological or tinctorial, the majority of present-day haematologists favour the conception that the platelets are formed from the megakaryocytes. However, as HAM (1953) stated in his textbook of histology: "The evidence for this view, though substantial, is not quite sufficient to justify closing one's mind to the possibility of one of the other views being correct".

In 1952 the enzyme acetylcholinesterase (AChE) was detected in the blood platelets (ZAJICEK & DATTA). Further studies demonstrated that in mammals this enzyme is distributed in inverse order between the erythrocytes and the platelets. Thus a diminishing erythrocyte AChE content in the

series from man, cow, guinea pig, horse, rat and rabbit to cat is accompanied by increased AChE activity of their respective platelets (ZAJICEK & DATTA 1953). This finding provided a new, biochemical approach to the problem of the origin of blood platelets.

The thiocholine method of KOELLE & FRIEDENWALD (1949) was adapted for histochemical tracing of AChE in the cells of blood-forming tissue (ZAJICEK, SYLVÉN & DATTA 1954). When the enzyme was thereby detected in the megakaryocytes, a diver method was elaborated (ZAJICEK & ZEUTHEN 1956) to determine quantitatively the enzyme activity content in individual megakaryocytes from various mammals.

The present paper summarizes the results reported in the earlier communications. Studies concerning the localization of AChE enzyme in platelets and megakaryocytes are also reported. A diver technique is described by which the characteristics of AChE found in rat megakaryocytes could be studied and compared with the properties of AChE from blood platelets of the same animal. The histogenesis of blood platelets and of the megakaryocytic system is discussed on the basis of biochemical evidence.

II. The Cholinesterase Activity of Erythrocytes and White Blood Cells.

The capacity of erythrocytes to split acetylcholine (ACh) was first described by GALEHR & PLATTNER (1928). Because of the physiologic significance of the acetylcholine-cholinesterase system, the properties of erythrocyte cholinesterase (ChE) have been studied in considerable detail. (For a survey of the literature, see AUGUSTINSSON 1948, 1950). This enzyme was found to resemble brain esterase, but to differ markedly from ChE found in some sera or in pancreas (literature reviewed by AUGUSTINSSON & NACHMANSOHN 1949). Following lysis of erythrocytes in distilled water, ChE remains attached to their stroma (BRAUER & BOOT 1945, PALEUS 1947, and AUGUSTINSSON 1948). The enzyme has a high affinity for ACh. No other ester — except acetylthiocholine (AThCh) — is hydrolyzed at a higher rate. The rate of hydrolysis of acetyl-β-methylcholine (MeCh) is higher than that of butyrylcholine (BuCh). The benzoylcholine (BzCh) and noncholine esters are hydrolyzed at a

very low rate or not all. If the enzyme activity is plotted against log M acetylcholine concentration, a bell-shaped curve is obtained. For this type of ChE the term acetylcholinesterase (AChE) has been suggested (AUGUSTINSSON & NACHMANSOHN 1949) to replace the earlier descriptions — "specific ChE" (GLICK 1945), "true ChE" (MENDEL & RUDNEY 1943), and "e type" of ChE (ZELLER & BISEGGER 1943).

The AChE activity of erythrocytes is very high in man, cow and guinea pig, but is low in rat, rabbit and cat (FEGLER, KOWARZYK & SZPUNNAR 1937, and AUGUSTINSSON 1950). For these differences in enzyme content no satisfactory explanation was given. A detailed study of the variations in erythrocyte AChE activity in human blood has recently been published by AUGUSTINSSON (1955).

Little attention seems so far to have been paid to the possible ChE content of blood cells other than erythrocytes. The available reports show a lack of conformity which may partly be attributed to present difficulties in finding satisfactory separation methods. FEGLER *et. al.* (1937) stated that no measurable amounts of ChE activity were detected in their experiments on white blood cells isolated from peripheral blood. COLLING and ROSSITER (quoted by ROSSITER & WONG 1949) found no significant correlation between the ChE activity of pathologic cerebrospinal fluid and the concentration of white blood cells. Similarly, GINSBERG, KOHN & NECHELES (1937), and PHILIPPU (1956) failed to detect ChE activity in polymorphonuclear leukocytes of empyemic pus from human subjects. The latter writer, however, found measurable ChE in white blood cells which were isolated from the peripheral blood (of man, dog, and, especially, rabbit). To explain these apparently conflicting findings, PHILIPPU advanced the theory that the enzyme is destroyed during the changes which the leukocytes, in particular the polymorphonuclear, undergo when they are transformed into pus cells. The presence of an MeCh-splitting enzyme in human leukocytes from peripheral blood was reported by DENNY & HAGERMAN (1956). In detailed investigations of white cells (approximately 95 per cent polymorphonuclear) from the peritoneal cavity of rabbit, ROSSITER & WONG (1949) demonstrated that these cells, even when concentrated to 150,000 per cu. mm, did not hydrolyze ACh, MeCh or BzCh. BRAUER & HARDENBERGH (1947) found little ChE activity in preparations obtained from the lymph nodes of

dogs and containing "mainly" lymphocytes. The ChE activity of lymphocytes and of thymocytes apparently is too low for histochemical demonstration with the thiocholine method (ROGISTER, DUMOULIN & GEREBTZOFF 1955). Similarly, no ChE activity was detected with a modified thiocholine method in the granulopoietic cells of man, rat and cat (ZAJICEK, SYLVÉN & DATTA 1954), even when these cells had been incubated for more than one hour. On the other hand, the same technique demonstrated ChE in blood platelets (cat) after only about 15 minutes' incubation.

III. The Cholinesterase Activity of Platelets.

1. *The properties of platelet cholinesterase*

ChE activity in platelets was first demonstrated in rat (ZAJICEK & DATTA 1952), and subsequently in cat, rabbit, dog and horse (AUGUSTINSSON, DATTA, GRAHN & ZAJICEK 1952). The characteristics of platelet ChE were studied in detail. Optimum enzyme activity was obtained at ACh concentrations between 1×10^{-3} M and 5×10^{-3} M. Higher ACh concentrations depressed the enzyme activity, and an almost symmetrical bell-shaped curve was obtained when esterase activity was plotted against the negative logarithm of the substrate concentration. Butyrylcholine (BuCh) was hydrolyzed at a much lower rate than ACh. Tributyrin (TB) and benzoylcholine (BzCh) were not hydrolyzed, but acetyl-β-methylcholine (MeCh) was split at a relatively high rate (AUGUSTINSSON *et. al.*). From these results in was concluded that platelet ChE is an AChE (aceto-cholinesterase, "specific", "true" ChE), known to be present in the erythrocytes of most animals (ZAJICEK & DATTA 1952, AUGUSTINSSON *et al.* 1952).

2. *Studies on the location of platelet cholinesterase*

As previously mentioned, the AChE of erythrocytes is localized to the cell stroma and remains attached to it even after lysis of the erythrocytes in water. It is generally held that the platelets undergo lysis in shed blood before becoming effective in coagulation (FLYNN & COON 1952). Detailed information on the process of platelet lysis, however, is still lacking. This may partly be ascribed to the prevailing concept of "rapid" disintegration of isolated platelets suspended in different

media (KIGUCHI & MIZUTA 1933, OLEF 1936, TOCANTINS 1938, and QUICK 1949), and further to inadequate methods for assay of platelet lysis. A colorimetric indicator is not present in platelets, in contrast to erythrocytes, in which haemoglobin forms the bulk of the dissolved material and serves as the best index in the assay of "haemolysis".

In earlier experiments with rat platelets (ZAJICEK & DATTA 1952) a precipitate or sediment was obtained by centrifuging the opalescent aqueous suspension of platelets, and protein material was found in the clear supernatant. The preliminary data suggested that AChE was associated with the sediment but not with the dissolved material in the supernatant. In the present paper this distribution of platelet material between the sediment and the water-soluble fraction is studied in greater detail. Morphologic characteristics of the sedimented material to which the AChE remains attached after "lysis" of the platelets in water are also investigated.

Method.

a. *Preparation of aqueous platelet suspensions:* Platelets were isolated from the blood of cat and rabbit in a manner similar to that previously described (ZAJICEK & DATTA 1952). The blood was drawn by heart puncture into a syringe containing 5 % sodium citrate solution (1:8). The platelets were separated by centrifugation, washed twice in saline and then suspended in a suitable volume of water with constant stirring. The suspensions were placed at 4° C for 2 to 3 hours. From the platelet preparations of each blood sample, a major portion of known volume was taken to obtain the insoluble matter or sediment as described below. The remainder was used for determinations of esterase, nitrogen and dry weight. The enzyme activity was measured in the Warburg apparatus (AUGUSTINSSON 1948). A 2.8×10^{-3}M solution of acetylcholine was used as substrate.
b. *Preparation of the sediment from the aqueous platelet suspension:* The sediment was obtained by centrifuging a known volume of the platelet suspensions at about 2,000 r.p.m. for approximately 20 minutes. Frequently, however, the supernatant fluid was not quite clear, having a pale blue, opalescent appearance. Repeat centrifugation of the supernatant at 9,000 r.p.m. for nearly one hour then brought down a smaller mass of sediment and left a more or less clear supernatant. The two sediments were combined and suspended in distilled water to the same volume as the original platelet suspension. Aliquots were taken for determination of esterase, nitrogen and dry weight.

c. *Ultrasonic treatment of platelet stroma:* Ultrasonic treatment was used to disintegrate the stroma of platelets in suspension*. Centrifugation at 3,000 r.p.m. for about 15 minutes then produced a dark grayish, more or less sticky sediment. The supernatant was pipetted off. The sedimented material was studied under the phase microscope. It was seen to consist of a mass of dark particles in Brownian movement, showing the morphologic characteristics of the granulomere part of the stroma. The supernatant was centrifuged for about one hour at 5,000 r.p.m. An almost transparent sediment was obtained which could readily be dispersed in water by gentle shaking. When inspected by phase microscopy this material showed the morphologic characteristics of the membrane-like material surrounding the granulomere.

Results.

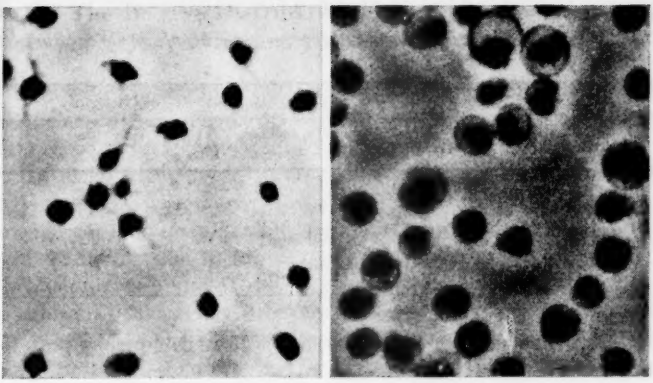
Table 1** shows that when blood platelets were suspended in distilled water, about 60 per cent of the total material and N content of the platelets went into solution, but that this supernatant contained no significant AChE activity. Practically all the enzyme activity of the original aqueous suspension of platelets was recovered from the sediment, which constituted only about 40 per cent of the mass of the original platelets.

Since the AChE was mainly bound to the sediment, the structure of this sediment was examined in order to ascertain how far it resembled the original platelets.

Standard light microscopy revealed no distinct structures. A drop of the sediment was then examined by phase-contrast microscopy under oil immersion; platelet-like structures were discerned. Platelets from the original plasma suspension were studied in the same manner. Figures 1 *a* and *b* show the findings from phase-contrast microscopy. The sediment from the aqueous suspension (figure 1 *b*) consisted of a mass of ballooned platelet-like corpuscles, indicating that some swelling had taken place. The pseudopodia-forming capacity and the tendency to adhere to the surface of the slide were lost. The corpuscles contained dark bodies which represented the remains of the granulomere of the normal platelets. The granulomere was surrounded by membrane-like structures corresponding to the hyalomere of intact platelets. These

* The disintegration of platelet stroma with ultrasonic treatment (700 kc/s. during 8 minutes with acoustic density about the cavitation threshold) was performed by Olle Lindström, of the Division of Physical Chemistry, the Royal Institute of Technology, Stockholm, according to his technique (Lindström 1955). No inhibition of AChE activity by ultrasonic treatment could be registered after the disintegration of platelet stroma.

** The experiments in table 1 were performed in collaboration with N. Datta.



a. *b.*

Fig. 1. a. Platelets in plasma. × 2,400.
b. Platelet stroma resuspended in distilled water. × 2,400.

Table 1.

Dry Weight, Nitrogen Content and AChE Activity of Aqueous Platelet Suspension, Stroma Material and Supernatant

	Platelets in water			Stroma material		
	Dry weight (mg)	N content (mg)	AChE (µl CO_2)	Dry weight (mg)	N content (mg)	AChE (µl CO_2)
Rabbit ♀	18.0 (100 %)	1.56 (100 %)	2,820 (100 %)	7.2 (40 %)	0.6 (38 %)	2,800 (99 %)
Rabbit ♀	13.2 (100 %)	1.44 (100 %)	2,400 (100 %)	5.4 (41 %)	0.55 (38 %)	2,280 (95 %)

	Supernatant		
	Dry weight (mg)	N content (mg)	AChE (µl CO_2)
Rabbit ♀	10.8 (60 %)	0.96 (62 %)	140 (5 %)
Rabbit ♀	7.8 (59 %)	0.88 (61 %)	190 (8 %)

AChE activity expressed in µl CO_2 evolved from substrate-bicarbonate solution during 30 minutes.

Table 2.

The AChE Activity of the Granulomere and Hyalomere of Platelet Stroma

Species	AChE activity per mg dry weight	
	Granulomere residue µl CO_2 per 30 min.	Hyalomere µl CO_2 per 30 min.
Rabbit ♀	150	460
Rabbit ♀	120	500
Cat ♂	180	620

corpuscles remained visible for many days in aqueous platelet suspensions.

To investigate if the AChE was localized to the residual granulomere or to the hyalomere, disintegration of the platelet stroma was accomplished with the aid of ultrasonic treatment. At subsequent differential centrifugation, two morphologically distinguishable fractions were separated from each other. The AChE activity in the granulomere and hyalomere of platelets from rabbit and cat is shown in table 2.

On a dry weight basis the enzyme activity of the stroma hyalomere was about 3 to 4 times that of the granulomere (table 2). Attempts to remove the AChE from the granulomere by subjecting this fraction to repeated ultrasonic treatment were unsuccessful.

Conclusions.

From these experiments it was concluded that when rabbit platelets are suspended in distilled water, roughly 60 per cent of the platelet mass is released into the water. The dissolved material apparently contains negligible amounts of AChE. The enzyme is bound — as in erythrocytes — to the platelet stromas, which remain suspended in the medium as pale, ballon-shaped corpuscles visible under the phase microscope. The residual granulomere portion is morphologically distinguishable from the hyalomere. On a dry-weight basis the hyalomere displays 3 to 4 times more AChE activity than the granulomere.

IV. The Inverse Distribution of Acetylcholinesterase between Erythrocytes and Platelets in Various Mammals.

It is well known that the erythrocytes of various mammalian species differ greatly in regard to their content of AChE (AUGUSTINSSON 1950). While human erythrocytes are highly active in splitting ACh, those of, for instance, rabbit contain little activity (FEGLER *et. al.* 1937). For this difference in enzyme content no plausible explanation was available.

In studying the AChE activity of platelets isolated from the blood of man and of rabbit, it was unexpectedly found that in rabbit — whose erythrocytes, as mentioned above, contain little AChE — the platelets were highly active. On the other hand, in man — whose erythrocytes are known to contain large amounts of AChE — the platelets were without activity. For better comprehension of this remarkable finding, investigation was also made of the platelet and erythrocyte AChE activity of other mammals, viz. cow, guinea pig, horse, rat and cat (ZAJICEK & DATTA 1953). The results are reproduced in table 3. The enzyme activity is expressed in μl CO_2 liberated from the substrate-bicarbonate solution per 1 mg nitrogen of platelet or erythrocyte stroma during 30 min. A 3×10^{-3} M acetylcholine chloride solution was used as the substrate and the measurements were made with the Warburg apparatus.

Table 3 shows that AChE is distributed in inverse order between erythrocytes and platelets. In man, AChE activity is concentrated to the erythrocytes and the platelets are devoid of activity. As the erythrocyte activity gradually decreases from man, cow, guinea pig, horse, rabbit and rat to cat, there is increased AChE activity in the respective platelets, until in cat almost all the enzyme is found there, the erythrocytes being practically without AChE activity.

The inverse distribution of the enzyme AChE between erythrocytes and platelets in various mammals (table 3) suggested a new, biochemical approach to the histogenesis of platelets. Like the red blood corpuscles, the platelets are not regarded as true cells, since they are non-nucleated. It was therefore assumed that the enzyme AChE is synthetized during the maturation process of the precursors of these bodies. A search for AChE in the blood-forming tissues might thus provide a clue to the genesis of platelets. For instance, if the theory of WRIGHT (1910) that the platelets arise by

Table 3.

The AChE Activity of Erythrocyte and Platelet Stroma from Different Animal Species

Species	AChE activity per mg N (µl CO_2 per 30 min.)			
	Erythrocyte stroma	Average	Platelet stroma	Average
Man ♂	2,400		0	
♂	2,050		0	
♀	2,100	*2,200*	0	*0*
Cow	1,880		60	
	1,900		70	
	1,960	*1,930*	30	*50*
Guinea-pig ♂	730		630	
♂	630		430	
♀	780	*710*	420	*490*
Horse ♀	820		1,240	
♀	640		1,740	
♂	420	*660*	1,540	*1,500*
Rabbit ♂	530		3,700	
♀	550		4,100	
♂	340	*470*	4,000	*3,930*
Rat ♂	290		3,700	
♂	305		5,050	
♂	320		3,000	
♂	180	*260*	5,200	*4,240*
Cat ♂	36		5,200	
♀	26	*30*	5,700	*5,450*

budding from the cytoplasm of megakaryocytes were correct, the human megakaryocyte must be devoid of AChE, whereas in cat the same cell must show fairly high enzymic activity. The megakaryocytes of other species should have activities between those of man and cat, in a pattern resembling that demonstrated in their platelet scale. The first investigations of this hypothesis were histochemical.

V. Histochemical Investigation of Acetylcholinesterase Activity in Bone Marrow Cells of Various Mammals.

Of the methods devised for histochemical demonstration of AChE activity, the thiocholine method most adequately satisfies the requirements for enzyme-substrate specificity. The thiocholine method of KOELLE & FRIEDENWALD (1949) was successfully employed in studies of AChE in tissue sections (*e. g.*

KOELLE 1951, COUTEAUX & TAXI 1952, GEREBTZOFF 1953). Attempts to demonstrate the presence of AChE in erythrocytes with this method were unsuccessful, however (COUTEAUX & TAXI 1952, HOLT & WITHERS 1952). Considerable difficulty was encountered in demonstrating AChE in the enzyme-bearing platelets, and reliable results were obtained only after modification of the procedure (ZAJICEK, SYLVÉN & DATTA 1954).

As devised by KOELLE & FRIEDENWALD (1949), the thiocholine method essentially involves two main steps of processing:

1 Incubation of fresh, frozen tissue sections in media containing acetylthiocholine (AThCh), or butyrylthiocholine (BuThCh), and copper glycinate. It is assumed that the enzymatically liberated thiocholine forms a precipitate of copper mercaptide at sites of enzyme activity.

2 The copper mercaptide is converted to a brown amorphous deposit of copper sulphide by treatment with dilute ammonium sulphide solution.

It was cursorily mentioned by KOELLE & FRIEDENWALD (1949) that presumed copper thiocholine appears as a white precipitate *in vitro,* but closer follow-up was not reported of such precipitate in tissue sections after incubation. When this end-product of the enzymatic reaction — recently shown to be Cu·thicholine sulphate (MALMGREN & SYLVÉN 1955) — was inspected under the phase microscope, it was observed to form needle-shaped crystals at or near sites of AChE activity. Since these crystals were visible on phase microscopy, a simple procedure for histochemical detection of AChE in bone-marrow cells was evolved, omitting the above-mentioned step 2, (ZAJICEK, SYLVÉN & DATTA 1954).

Histochemical Procedure.

Three or four drops of KOELLE'S incubation medium were placed on a slide, mixed with a small drop of washed bone-marrow cell suspension and covered with a coverslip. The edges of the slip were sealed with vaseline to prevent evaporation. The formation of crystals was studied under the phase microscope. When individual cells were tested, a drop of paraffin oil was placed on a slide. A small amount (about 0.05 μl) of incubation medium together with a cell was then introduced under the oil with the aid of a braking pipette. The cell was isolated and placed in the incubation medium according to the description given in a previous paper (ZAJICEK & ZEUTHEN 1956).

Table 4.

The AChE Activity of Erythrocyte-Erythropoietic and Platelet-Megakaryocytic Systems in Various Mammals

Species	Erythropoietic cells Histochemical data	Erythrocytes	Platelets	Megakaryocytes	
		Manometric data* µl CO_2 per mg dry weight per hour		Histochemical data**	Diver method*** 10 6 µl CO_2 per hour
Man	+ + +	460	0	0	0
Guinea-pig	+ +	160	100	+	150
Rat	—	60	520	+ +	1,000
Cat	0	6	960	+ + +	2,000

* Zajicek & Datta 1953, ** Zajicek 1954, *** Zajicek 1956.

Results.

This thiocholine technique showed that the distribution of AChE between platelets and erythrocytes (table 3) is preceded in the bone marrow by a similar distribution pattern in the erythropoietic cells and megakaryocytes (Zajicek, Sylvén & Datta 1954, Zajicek 1954). The results are summarized in table 4, which also gives manometric data for AChE activity of the erythrocytes and platelets of the respective animals.

In the bone marrow of cat, no indication of enzymatic hydrolysis of AThCh was detected in the erythropoietic cells, even after incubation for about 1 hour. Negative results were also obtained when the erythropoietic cells were disrupted with the aid of a micromanipulator and the nuclear substance was exposed. The granulopoietic cells were similarly negative. The only cell system in cat bone marrow which displayed ChE activity was the megakaryocytic system. In this cell system haematologists usually distinguish 3 stages of maturation: *1* Megakaryoblast, the earliest known precursor cell of the system. ("Er ist myeloblastenähnlich aber grösser als dieser" Rohr 1949). *2* Promegakaryocyte. *3* Mature megakaryocyte. The enzyme activity of megakaryocytes and promegakaryocytes became evident immediately after incubation for 5 to 15 minutes, and that of the nearest precursor cell (megakaryoblast, 18 to 24 μ in diameter) after 20 to 30 minutes. When the incubation period was prolonged from 30 to 50 minutes, crystalline deposits were observed in a few cells with diameter smaller than a megakaryoblast. The possibility was suggested

that these cells are precursors of the megakaryoblast (ZAJICEK 1954). When BuThCh was used as the substrate, the histochemical reaction of the megakaryocytes was scarcely detectable, even after incubation for 1 hour, indicating that BuThCh was split at a very low rate. This suggested that the ChE present in cat megakaryocytes is an AChE.

As seen from tables 3 and 4, the platelets in cat displayed high AChE activity. The absence of this enzyme in the cytoplasm and in the nucleus of cat erythropoietic cells, therefore, showed it highly unlikely that platelets derive from the normoblast nucleus. The finding that in cat bone marrow only the megakaryocytic system exhibited AChE activity, on the other hand, gave strong support to the view that platelets arise from megakaryocytes. It is also noteworthy that the histochemical demonstration of AChE in megakaryocytes and in platelets isolated from peripheral blood required the same incubation periods (ZAJICEK 1954).

From human subjects, whose erythrocytes contain large amounts of AChE, only very dilute bone-marrow suspensions were investigated and, in control experiments, only single isolated cells, so as to avoid diffusion artefacts. The erythropoietic cells gave a highly positive histochemical reaction (ZAJICEK, SYLVÉN & DATTA 1954). Tables 3 and 4 show that no AChE was demonstrated in human platelets. Nor was any activity detected histochemically in megakaryocytes (ZAJICEK, SYLVÉN & DATTA 1954, ZAJICEK 1954). This identical behaviour of human platelets and megakaryocytes constitutes further evidence of the megakaryocytic origin of the platelets. Support was likewise obtained from the findings in rabbit, rat and guinea-pig blood, in which megakaryocytic activity followed the same trend as that of their respective platelets (ZAJICEK 1954).

Both processing steps of the thiocholine method (KOELLE & FRIEDENWALD 1949) were employed by ROGISTER (1954 a) for detection of AChE activity in platelets and erythrocytes in blood smears. ROGISTER concluded that his histochemical results corroborated the manometric data in table 3, showing high AChE activity in the platelets of mammals whose erythrocytes contain little activity and, *vice versa,* absence of such activity in human platelets. In a separate paper, ROGISTER (1954 b) reported the presence of AChE in megakaryocytes of rat, cat, mouse, rabbit and guinea pig, and stressed that the

constituents of the megakaroycytic system exhibited histochemically variable degrees of AChE activity, probably reflecting different stages of maturation. Very recently, ROGISTER (1956) confirmed the absence of a histochemical reaction for AChE in human megakaryocytes (ZAJICEK, SYLVÉN & DATTA 1954, ZAJICEK 1954). Investigations of the AChE activity in the platelet-megakaryocytic system of cattle, using the thiocholine method (KOELLE 1951) and its modification (ZAJICEK, SYLVÉN & DATTA 1954), were reported by WINQUIST (1955). The effects of ionizing irradiation on the AChE in the megakaryocytes of mice were studied by HAJDUKOVIC (1955).

Cytoplasmic nucleotide substances and dry mass were determined in megakaryocytes in different developmental stages by DATTA, THORELL & ÅCKERMAN (1955). Phosphatases were reported by STORTI (1951) to be present in the megakaryocytes of man, but by WINQUIST (1955) to be absent in the megakaryocytes of cattle. The similarities in the content of glycogen and of polysaccharide granulation in platelets and megakaryocytic cytoplasm under normal and pathologic conditions were studied in detail by STORTI, PERUGINI & SOLDATI (1953), and by HECKNER (1957).

Comments on the localization of acetylcholinesterase in megakaryocytes

The thiocholine method for histochemical detection of AChE in cells, while satisfying the requirements for enzyme specificity, also has a serious drawback. As mentioned in the beginning of this chapter, the detection of enzymatic activity in cells is based on the visibility to phase microscopy of crystalline deposits of CuThCh formed during enzymatic reaction (ZAJICEK, SYLVÉN & DATTA 1954). Several factors other than this activity influence the amount of CuThCh deposited in various sites. These factors are the Cu^{++} ion concentration, the period of incubation, convection currents due to temperature differences in the incubation medium, and the rate of diffusion of CuThCh. It was therefore suggested that only the first-formed crystalline nuclei may reliably indicate the enzyme sites (MALMGREN & SYLVÉN 1955). With the thiocholine method in its present form, no indication of the intracellular distribution of AChE can be obtained in small cells such as the normoblast, since activity in these cells is readily detectable only

when crystals protrude from them, or when a prolific deposit of crystals surrounds them.

Some inference, however, may be drawn from large cells such as megakaryocytes. In cat bone marrow, megakaryocytes are occasionally found which have a small (about 20 μ in diameter), eccentrically placed nucleus and a large (50 to 60 μ) cytoplasmic body. When such cells, or even a fraction of the cytoplasm dissected from the cell with a micromanipulator, were incubated (ZAJICEK, unpublished data), granular and rod-shaped deposits within the cytoplasm became clearly visible after only a few minutes. Using the modified thiocholine technique (ZAJICEK, SYLVÉN & DATTA 1954), WINQUIST (personal communication, 1954) obtained similar results in intact cat megakaryocytes, and also in the megakaryocytes of cattle (WINQUIST 1955). ROGISTER(1956) also concluded that there could be no doubt of the localization "exclusivement cytoplasmique de l'activité enzymatique observée dans le mégakaryocyte".

The observation that the AChE of megarkaryocytes is localized to the cytoplasm is in conformity with Wright's theory that platelets derive from the cytoplasm of megakaryocytes. The data in table 2 of the present report show that in platelets the AChE is highly concentrated in the almost transparent structures of platelet stroma surrounding the granulomere (figure 3). In recent electron microscopic studies (KAUTZ & DE MARSH 1955), round or ovoid granules of considerable electronic density surrounded by cytoplasmic matrix with poorly defined density were demonstrated in megakaryocytes. Assuming the derivation of platelets from the cytoplasm of megakaryocytes, and provided that during this process no translocation of AChE molecules takes place, it may be pertinent to suggest that the AChE in mature megakaryocytes may also be largely localized to the cytoplasmic matrix surrounding the granules.

The histochemical approach yields no information concerning possible AChE activity in the nuclei of developing megakaryocytes. When the maturation process of megakaryocytes is accomplished, the cytoplasmic mass separates from the nucleus by a process at present poorly understood. The remaining "naked" and apparently degenerated nuclei — frequently observed in cell suspensions prepared from mouse bone marrow — were subjected to histochemical testing, but showed no indication of AChE activity (incubation time 40 min).

VI. Quantitative Studies on Acetylcholinesterase Acitivity in Single Megakaryocytes.

The histochemical investigations clearly demonstrated the presence of AChE in megakaryocytes from mammals whose platelets also contain AChE, and the absence of such activity in megakaryocytes from man, whose platelets lack this enzyme. The histochemical data further suggested that in mammals with low platelet AChE activity, the megakaryocytes contained little AChE, and *vice versa* (ZAJICEK 1954). This histochemical evidence, however, required confirmation by experiments performed on a quantitative basis.

Because of the lack of a satisfactory technique for mass isolation of megakaryocytes, their AChE could not be studied by standard titration or by Warburg's manometric method. To measure in the Warburg apparatus the enzymatic activity of megakaryocytes with a diameter of 50 to 60 μ from rat or guinea pig, at least 20,000 and 100,000 cells, respectively, are required. The total content of megakaryocytes at this stage of maturation in one femur of adult albino rat is only about 1,000 (ZAJICEK, unpublished data). Hence it is evident that a micromethod was essential for quantitative study of the enzymatic activity of megakaryocytes.

The most sensitive method hitherto evolved for ChE determination is the Cartesian diver microgasometer of LINDERSTRØM-LANG (1937). With this apparatus analysis was made of serum samples (LINDERSTRØM-LANG & GLICK 1938) and tissue fragments with ChE activity corresponding to the liberation of about 0.01 to 0.1 μl CO_2 per hour (BOELL & SHEN 1950). Recently, a further development of the Cartesian diver technique for ChE determination was described, which permitted quantitative determination of enzymatic activity corresponding to the liberation of 10^{-4} μl CO_2 per hour with an accuracy of about $\pm$ 5 per cent (ZAJICEK & ZEUTHEN 1956). With this technique the amount of AChE activity in a single ganglion cell (GIACOBINI & ZAJICEK 1956) and in a single cell of the megakaryocytic system (ZAJICEK & ZEUTHEN 1956) could be measured.

1. *Determination of rates of synthesis of acetylcholinesterase during the maturation of megakaryocytes*

During maturation the megakaryocytes divide by mitosis (JAPA 1943), but the nuclear divisions are not followed

by corresponding separation of the cytoplasmic mass. Consequently, the megakaryocytes continuously increase in size throughout maturation. In unstained preparations of bone marrow, those cells of the megakaryocytic system which have reached a diameter of about 20 to 60 μ can be distinguished from the other cell systems present. Since individual megakaryocytes also can readily be isolated and placed in divers, it was suggested that this cell system constituted an ideal medium for studying the synthesis of a specific enzyme during maturation of the somatic cells (ZAJICEK 1956 b).

The increase in AChE activity during megakaryocyte maturation was studied in cells isolated from rat bone marrow (ZAJICEK 1956 b). It was shown that cells with diameter ranging from 24 to 45 μ displayed wide variations in enzyme content. Most cells were highly active, but some showed remarkably low AChE activity. When the megakaryocytes had attained a diameter of about 45 μ, their content of enzyme was invariably increased and its fluctuations were relatively small. Such large cells were therefore selected for comparative studies of the AChE content of megakaryocytes from various mammalian species.

2. *Determination of acetylcholinesterase activity in large megakaryocytes of various mammals*

Megakaryocytes from cat, rat, guinea pig and man were investigated (ZAJICEK 1956 a). The results are reproduced in the final column of table 4. The highest megakaryocyte AChE activity was found in cat, which also showed the richest platelet content of the enzyme. A single megakaryocyte from cat bone marrow evolved about $2{,}000 \times 10^{-6}$ μl CO_2 per hour from bicarbonate buffer. This corresponds to the hydrolysis of about 2.4×10^{-2} μg AThCh. In rat and guinea pig the megakaryocyte activity was much lower, and followed the trend previously described in the platelets of these species. In man the megakaryocytes and platelets were without AChE activity.

The capacity of megakaryocytes to split AThCh in these quantitative studies was considered to indicate the presence of AChE. This assumption was permissible on the basis of evidence obtained in the preceding histochemical experiments. Further arguments for this view, obtained from studies with the diver technique, are presented below.

3. *Studies on the properties of acetylcholinesterase in megakaryocytes*

When differentiation between ChE and AChE is quantitatively attempted on the macroscale (*e. g.* in the Warburg apparatus), equal samples of the material under investigation are taken from a stock solution previously prepared and tested against specific inhibitors, various substrates and various concentrations of the same substrate (AUGUSTINSSON 1948). It was shown in diver experiments that megakaryocytes of apparently similar morphologic characteristics display wide variations in their capacity to hydrolyze AThCh (ZAJICEK 1956 b). Similarly, some ganglion cells showed very high activity (2×10^{-3} μl CO_2 per hour), while others contained little or no measurable activity (GIACOBINI 1957).

Obviously, the effect of specific inhibitors and the hydrolysis rates of various substrates are very difficult to evaluate when the measurements are performed on different cells containing the enzyme in dissimilar quantity. To obtain reliable results, therefore, it was essential to make at least two measurements on the same cell. The diver technique previously described (ZAJICEK & ZEUTHEN 1956) permitted only single measurements on individual cells. A new procedure for making, handling and charging the diver was therefore elaborated. This made possible two determinations on the same cell, *e. g.* in the presence and in the absence of inhibitor, or first with substrate A and subsequently with substrate B.

Method.

Divers were pulled from thin-walled capillary (0.5 to 1 mm in diameter) according to the sketch in figure 2. In the preceding experiments it was found that divers pulled from Jena glass, filled with gas bubble (5 per cent CO_2 and 95 per cent N_2) and bicarbonate solution saturated with this gas, became constantly heavier even after 24 hours or more, probably due to absorption of CO_2 by the glass walls. This was not observed to occur in divers made from Phoenix glass (ZAJICEK & ZEUTHEN 1956). Since absorption of CO_2 by the diver walls caused serious experimental difficulties, Phoenix glass is now used for making the divers. The glass is heated from one side when the "tip" is made and from the other when the "tail" is pulled. When the diver is broken off from the capillary, its tail is excessively long. A small amount of water is then introduced *via* the tip, and the diver is submerged in water in a special vessel (ZAJICEK & ZEUTHEN 1956), where it is tested for buoyancy. The

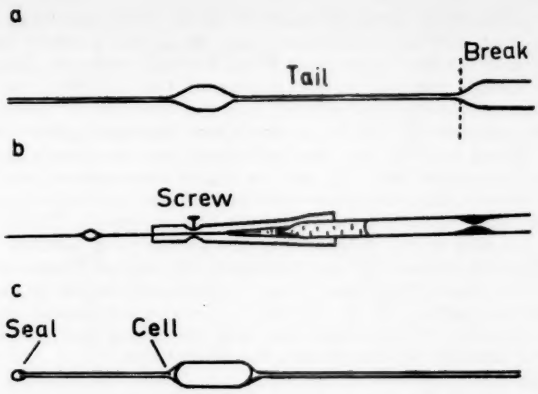


Fig. 2. The diver is pulled from a thin-walled capillary (a), broken off, and its tail firmly fastened into rubber tubing placed over a braking pipette (b). The diver is charged (c) with bicarbonate buffer solution (in the tail), with CO_2-N_2 gas mixture (in ampulla) and with a cell surrounded by substrate-bicarbonate buffer solution. The tip is sealed with wax.

long tail renders the diver overweight, and buoyancy is regulated by progressive shortening of the tail. For the ChE experiments only divers buoyant with the minimum liquid charge were used. Such a diver is shown in figure 2 c.

To charge the diver with the cell, the tail was inserted into a fine rubber tubing (inner diameter about 200 μ), which was also fitted over a braking pipette containing bicarbonate buffer solution saturated with a mixture of 5 per cent CO_2 and 95 per cent N_2. By compressing the lumen of the rubber tubing with a screw, the diver was held firmly in position. Bicarbonate buffer solution was then sucked into the diver, followed by a bubble of the gas mixture (cf. ZAJICEK & ZEUTHEN 1956). The bubble almost filled the volume of the ampulla and was succeeded by more bicarbonate buffer. The liquid in the diver tip was expelled and the narrowest part of the tip was filled with substrate-bicarbonate solution. The composition of the bicarbonate solution used was stated in a previous paper (ZAJICEK & ZEUTHEN 1956). The cell, already deposited in substrate solution on a hollow slide, was finally sucked into the diver with the smallest possible amount of the surrounding liquid, and was immediately followed by gas-saturated substrate solution. The diver tip was sealed with wax and the enzymatic activity was measured as previously described (ZAJICEK & ZEUTHEN 1956).

When the enzymatic hydrolysis of the substrate had been determined, the diver was removed from the manometer and the tail was fastened into the rubber tubing as before. A new substrate, or a mixture of substrate-inhibitor, was now carefully sucked into the tip, ampulla and tail. It was found that the megakaryocyte, when once

placed in the diver, remained attached to the glass wall when a new medium was carefully introduced into the diver. Contact between the cell and the gas bubble had to be avoided, however, since such contact usually resulted in detachment of the cell from the diver wall. The gas bubble was sucked into the diver after the medium. For this purpose the tip of the diver was fastened tightly into the rubber tubing and the gas was introduced *via* the diver's tail. The tip was then sealed with wax and the second measurement was made.

It is obvious that the above-described process can only be applied to cells like megakaryocytes, in which the enzyme is firmly bound to the cell and is not released into the surrounding medium during the initial experiment. If the possibility of enzyme release (by diffusion or because of disintegration of cytoplasm) cannot be excluded, the first medium should be retained in the diver. A second substrate or an inhibitor-substrate solution may be added and mixed with the first medium by the process described below.

In the experiments with megakaryocytes, only the effect of introducing an inihibitor was investigated. A known amount of inhibitor in the substrate solution, saturated with the gas mixture, was placed on a slide under paraffin oil. The diver's tail was inserted into the rubber tubing, the seal on the tip was broken, and the inhibitor was sucked into the diver. The paraffin oil saturated with gas mixture was next introduced into the diver until the oil meniscus approached the vicinity of the cell. In this way the first medium and the subsequently introduced substrate-inhibitor solution reached the ampulla and mixed. When the oil meniscus had been moved two or three times between the diver tip and the cell, the tip was sealed with wax. The diver was then replaced in the apparatus for a second measurement of enzymatic activity.

Results.

The non-enzymatic hydrolysis of the esters employed was first determined. The experiments were performed at 23° C. The divers were charged with about 0.4 μl of ester-bicarbonate solution saturated with a mixture of 5 per cent CO_2 and 95 per cent N_2. The calculated values for the non-enzymatic hydrolysis of 0.05 μl of acetylcholine chloride (ACh), acetylthiocholine iodide (AThCh) and butyrylcholine iodide (BuCh) in the concentration range 3.5×10^{-3} to 100×10^{-3} M are shown in figure 3. It is seen that the spontaneous hydrolysis of AThCh (crosses) was lower than that of ACh (open circles) and that the former was therefore better suited for diver work. It must be mentioned that in divers charged with only 0.05 to 0.1 μl, which was the amount of substrate-bicarbonate solution used in the AChE experiments, the measured spontaneous hydrolysis of the substrate will be much lower than could be expec-

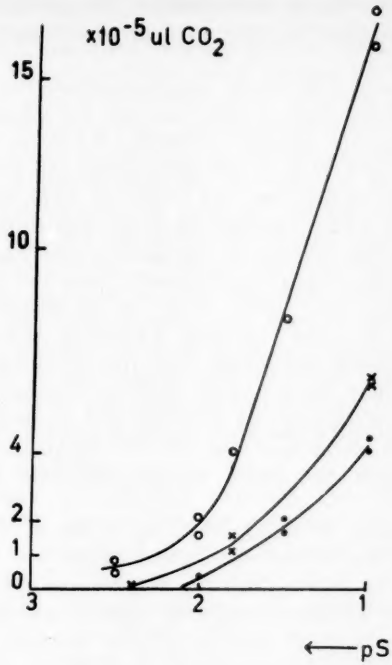


Fig. 3. Nonenzymatic hydrolysis of 0.05 µl of acetylcholine chloride (open circles), acetylthiocholine iodide (crosses), and butyrylcholine iodide (filled circles) bicarbonate buffer solution in one hour as a function of the concentration at 23° C. pS = — log molar concentration.

ted from the data in figure 3. In such divers only part of the total substrate is present in the vicinity of the gas bubble, the remainder being in the long narrow capillary far distant from the gas phase. The diffusion path for CO_2 formed by spontaneous hydrolysis of ACh is therefore very long (cf ZAJICEK & ZEUTHEN 1956, p. 575). In control experiments with divers weighing about 1 mg and charged with 0.05 µl of a 5×10^{-3} M ACh solution, the spontaneous hydrolysis of this substrate was below the sensitivity of the method during the first 6 hours of the experiments.

The non-enzymatic hydrolysis of 0.4 µl of acetyl-β-methylcholine iodide (MeCh) and benzoylcholine chloride (BzCh)

was also tested with the diver method. No spontaneous hydrolysis was registered, however, even at concentrations as high as 20×10^{-3} M.

In experiments with single megakaryocytes (40 to 50 μ in diameter) isolated from rat bone marrow, the enzymatic hydrolysis of one of the substrates BuCh, BzCh, or MeCh (concentration 5×10^{-3} M) was first determined and was then compared with the result from the same cell when the substrate was 5×10^{-3} M AThCh or ACh solution. The findings showed that the esterase present in megakaryocytes has properties similar to those of AChE in platelets from the same animal (AUGUSTINSSON *et al.* 1952). None of the esters tested was hydrolyzed at a higher rate than AThCh or ACh. The hydrolysis rate of MeCh was higher than that of BuCh; the latter rate was very low. BzCh was not split. A more detailed quantitative report of the results obtained will be published later, together with determinations of the enzymatic hydrolysis of MeCh, BuCh and BzCh at concentrations higher than 5×10^{-3} M.

In experiments with inhibitor, the effect of Mipafox (bismonoisopropylamino fluorophosphine oxide) was investigated. It was recently shown (ALDRIDGE 1953, and DAVISON 1953), that 8×10^{-7} M Mipafox completely inhibits nonspecific serum ChE, but affects AChE activity only in concentrations higher than 6×10^{-6} M. In the inhibitor experiments the hydrolysis of AThCh by the megakaryocyte in the absence of inhibitor was first determined. A Mipafox (3×10^{-6} M) substrate solution was then introduced into the diver and the enzymatic activity of the megakaryocyte in its presence was determined. With this Mipafox solution, no noteworthy inhibition of enzymatic activity was registered, which suggested the presence of AChE in the megakaryocyte.

The curve showing the relationship between esterase activity and substrate concentration, which was obtained in macroscale experiments with the Warburg apparatus on platelets (AUGUSTINSSON *et al.* 1952, and ZAJICEK & DATTA 1952), could not be drawn for single megakaryocytes. This was partly because of technical obstacles to performing more than two experiments on the same cell, and partly because the spontaneous hydrolysis of the amounts of AThCh and ACh solutions used approached at higher concentrations the expected values for the enzymatic activity of the cell (figure 3).

VII. Discussion.

Since AChE was first demonstrated in human erythrocytes (GALEHR & PLATTNER 1928), much interest has centred round the question of this enzyme's precise function as regards the erythrocytes. GREIG & HOLLAND (1949) observed that inhibitors of ChE produced an increase in the permeability of the red cells. They suggested that the maintenance of the integrity of erythrocytes depends in some way on the activity of their AChE. The findings concerning the inverse order of distribution of AChE between erythrocytes and platelets (chapter IV), and the apparent lack of measurable AChE activity in the erythrocytes or platelets of some mammalian species (table 4), limit the possible role of AChE in maintaining the integrity of these cells. It was instead suggested that erythrocytes and platelets are to be regarded only as carriers of this enzyme, the presence of which in the circulation is doubtless of importance (ZAJICEK 1956).

The average ACh-splitting capacity (at 25° C and at 3×10^{-3} M ACh) per erythrocyte from man and per platelet from cat were calculated to be of the same order of magnitude — about 6×10^{-6} μg per hour (ZAJICEK, unpublished data). The life span of erythrocytes, however, is estimated to be about 100 days (ROHR 1949, p. 141) and that of blood platelets only about 4 to 5 days (TOCANTINS 1938, MAUPIN 1954, p. 46). As AChE in man is confined to the erythrocytes, this enzyme is transported at least 20 times longer in the human circulation than in cat blood, where it is contained in the platelets. Because of the consequent numerical superiority of erythrocytes (cf. p. 26, 4.), the amount of AChE in circulating blood became more than 20 times higher in man than in cat. The high AChE activity of cow and guinea-pig blood and the contrasting low activity of rabbit blood (MENDEL *et al.* 1943) would seem to be explainable on the same basis (table 3). This is doubtless a striking illustration of how, in mammals, wide variations in the amount of an enzyme in circulation may arise during evolution simply by "transfer" of this enzyme synthesis from the precursors of the shorter-lived platelets to the precursors of longer-lived erythrocytes, or *vice versa.*

In investigations of the blood-forming tissue it was found that the erythropoietic cells gave a positive histochemical reac-

tion in mammals whose erythrocytes were highly active, but were "negative" in species whose enzyme activity was concentrated to the platelets (chapter V). The only cell system which displayed the same activity pattern as the circulating platelets in the various species was the megakaryocytic. This was clearly demonstrated by a histochemical technique (chapter V) and also by a quantitative approach with a diver method (chapter VI). It was therefore concluded that the megakaryocytes are the only possible bone-marrow cells from which the platelets can originate. Further evidence for the megakaryocytic origin of blood platelets was provided by the demonstration (chapter VI) that the ChE associated with rat megakaryocytes had properties similar to the AChE earlier found (AUGUSTINSSON *et al.* 1952) in the platelets of the same species.

Very little is known of the derivation of megakaryocytes. Studies hitherto reported were mainly morphologic and their results were not in agreement. Some of the expressed opinions are cited below. HOWELL (1890) believed that the megakaryocytes resulted from the growth of small lymphoid cells. JORDAN (1918) suggested that they were formed by hypertrophy of haemoblasts. DI GUGLIELMO (1925) advanced the theory that megakaryocytes originated from fusion of other cells – prepolykaryocytes. DOWNEY, PALMER & POWELL. (1930) considered that megakaryocytes derived from the myeloblast, or directly from the reticular stellate cells of the liver sinusoids. POTTER & WARD (1941) maintained that megakaryocytes originated from the haemocytoblast which they considered to be "the last developmental stage that the megakaryocyte has in common with other cells of both normal and extramedullary myeloid and lymphoid tissues".

The observations concerning an inverse order of AChE distribution between the erythropoietic cells and the megakaryocytes of various mammalian species (table 4) opened up a biochemical approach to the problematic histogenesis of the megakaryocytic system. An evaluation of the data relevant to this distribution of AChE will therefore be attempted.

The lack of demonstrable AChE in the erythropoietic system or in the megakaryocytes of some mammals suggested that this enzyme is not involved in any of the basic metabolic processes connected with the existence of these cells. AChE is apparently synthetized during their maturation process,

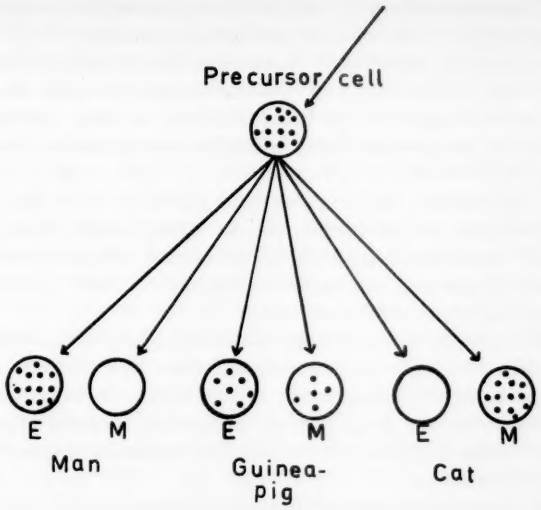


Fig. 4. Schematic presentation of the suggested distribution between erythroblast (E) and megakaryoblast (M) of the factors (dots) responsible for AChE synthesis during the maturation of erythropoietic cells and megakaryocytes. In the final differential mitosis of the postulated common precursor cell, all the factors are transmitted to the erythroblast in man. The erythroblast receives most, but the megakaryoblast also some, in guinea-pig. The factors are almost exclusively transmitted to the megakaryoblast in cat. The possible transmission of minute amounts of the factors to cat erythroblast (see table 3) could not be indicated in this diagram for technical reasons.

in order to perform its function later when carried in the circulation by the erythrocytes and/or platelets. The results in tables 3 and 4 clearly show that high enzyme synthesis in one system implies lower enzyme synthesis in the other. To explain this circumstance, the working hypothesis was advanced that the erythropoietic cells and the megakaryocytes derive from a common cell to which, during the differentiation process, the factors responsible for later AChE synthesis are conveyed (figure 4). During the final differential mitosis these factors (figure 4, dots) are transmitted to the erythropoietic system in man, to the erythropoietic and megakaryocytic systems in cow, guinea pig (mostly to the former system), horse, rat, mouse and rabbit (mostly to the latter system), and almost exclusively to the megakaryocytic system in cat (ZAJICEK 1956). During the maturation of erythropoie-

tic cells and megakaryocytes, AChE is synthetized in amounts proportionate to the number of factors received.

This working hypothesis of the common origin of the erythrocyte-erythropoietic and platelet-megakaryocytic systems was further suggested by the following striking similarities between the properties of erythrocytes and platelets (ZAJICEK 1956):

1. In birds the erythrocytes and platelets (thrombocytes) are nucleated. In mammals, on the other hand, these blood elements are non-nucleated. Mammalian erythrocytes and platelets, therefore, are to be considered only as cytoplasmic products of their precursor cells.

2. The osmotic behaviour of platelets greatly resembles that of erythrocytes (DATTA & ZAJICEK 1954). In hypotonic saline solution both cell types swell and within a certain range of salt concentration lysis begins. Following complete lysis of platelets and erythrocytes in distilled water, stromas remain in suspension.

3. These erythrocyte and platelet stromas retain the entire AChE activity of the original elements (AUGUSTINSSON 1948, and present study, chapter III).

4. Erythrocytes and platelets are present in circulating blood in large numbers as compared with other blood elements. In man the ratio of erythrocytes to platelets is about 20 to 1. The life span of human erythrocytes (ROHR 1949) is presumed to be about 20 times longer than that of platelets (TOCANTINS 1938, and MAUPIN 1954). The two blood elements would therefore seem to have parellel rates of production in man. It is also noteworthy that in man, whose erythrocytes contain AChE, the average enzymatic activity of a single erythrocyte stroma is of the same order of magnitude (page 23) as the activity of a single platelet stroma in cat, in which the platelets contain the enzyme.

The data presented in this study yield no indication of the possible nature of those factors (figure 4, dots) which determine the amount of AChE synthetized during the maturation process of erythropoietic cells and megakaryocytes. They may be some precursor molecules of the enzyme, or template molecules responsible for enzyme formation, or even active AChE molecules already present in the precursor cell and which, after transmission to the erythroblast or megarkaryoblast during maturation, induce their own multiplication in

proportion to their original number. When the synthesis rates of AChE during the maturation of cat and guinea-pig megakaryocytes (ZAJICEK, unpublished data) were compared with those obtained for rat megakaryocytes (ZAJICEK 1956 b), it was found that cat megakaryocytes, even in their earliest maturation stage (megakaryoblasts with diameter 20 to 24 μ), exhibited higher enzymatic activity than megakaryoblasts from rat which, in their turn, were many times more active than guinea-pig megakaryoblasts. This showed that species differences concerning the AChE content of megakaryocytes exist in the earliest known maturation stages of the megakaryocytic system. Whether or not active AChE molecules are present in the precursor cells of the erythropoietic or the megakaryocytic system remains unknown, however.

In postnatal blood formation the megakaryoblasts (like the proerythroblasts in the erythropoietic system) are regarded by many haematologists as the stem cells of the megakaryocytic system (ROHR 1949). In experiments on cat bone marrow (ZAJICEK 1954), in which the AChE is concentrated to the megakaryocytic system, the presence of AChE was histochemically demonstrated in a few cells much smaller even than the megakaryoblast which, according to ROHR (1949), is "myeloblastenähnlich aber grösser als dieser". The identification of these small cells has not yet been accomplished. They may represent only an earlier stage of the megakaryoblast, or a stem cell of the megakaryocytic system, or possibly the postulated common, enzyme-bearing stem cell of both the megakaryocytic and the erythropoietic system (figure 4).

In the earliest stages of prenatal blood formation in the yolk-sac of pig embryo about 10 mm in length, the erythroblasts were shown to arise from "metamorphosing and separating" endothelial cells of the yolk-sac blood vessels (JORDAN 1918). At this stage leukocytes are still absent, but megakaryocytes are present in relatively large numbers (JORDAN 1918). From the working hypothesis that the erythroblast and megakaryoblast derive from a common cell — advanced to explain the observed transfer of AChE synthesis from erythropoietic cells to megakaryocytes or *vice versa* (figure 4) — it would be expected that in the yolk-sac the megakaryocytes arise during the differentiation process of the endothelial cells towards erythroblasts. This mode of origin of megakaryocytes was, in fact, described by JORDAN (1918), and had earlier been

indicated by WRIGHT (1910) in his paper on the histogenesis of blood platelets, when he wrote that in the yolk-sac of guinea-pig embryo about 4.5 mm long, some at least of the small megakaryocytes "seem to be formed by transformation of endothelial cells of blood vessels". From the findings in the present study it would appear that histochemical techniques for tracing the AChE activity in the earliest stages of blood formation in the yolk sac of various mammals may later help to elucidate the differentiation process of the endothelial cells towards erythropoietic and megakaryocytic systems.

Summary.

Blood platelets contain a cholinesterase with properties similar to those of acetycholinesterase (AChE) present in erythrocytes of most mammals. The enzyme remains attached to the platelet stroma after lysis of the platelets in distilled water. It was found to be highly concentrated to the hyalomere (chapter III).

In man, cow, guinea pig, horse, rabbit, rat and cat, AChE is distributed between erythrocytes and platelets in an inverse order. In man, only the erythrocytes contain the enzyme and the platelets are without activity. In the other species a decreasing erythrocyte AChE activity is accompanied by increasing platelet activity, until the opposite extreme is reached in cat. Here the enzyme is concentrated to the platelets, the erythrocytes being practically devoid of AChE (chapter IV).

The thiocholine method of KOELLE and FRIEDENWALD was adapted for histochemical tracing of AChE in the cells of blood-forming tissue. It was thereby shown that the observed distribution of AChE between the erythrocytes and the platelets of various mammals is preceded in the bone marrow by a similar distribution between erythropoietic cells and megakaryocytes. The megakaryocytes, in which the enzyme was located to the cytoplasm, were demonstrated to be the only cell system which in various mammalian species displays an AChE activity pattern similar to that of platelets isolated from the circulation. It was therefore concluded that the megakaryocytes are the only possible cellular origin of the platelets (chapter V).

A diver method was elaborated to determine quantitatively the enzyme content of individual megakaryocytes. The correlation between the AChE activity of platelets and megakaryocytes from different mammals, first demonstrated by a histochemical technique, was confirmed in quantitative experiments with divers. The properties of the AChE in rat megakaryocytes were also studied. The enzyme in megakaryocytes was found to display characteristics similar to the AChE in platelets from the same animal. The rate of synthesis of AChE during the maturation of megakaryocytes was investigated in cells isolated from rat bone marrow (chapter VI).

The findings concerning "transfer" of AChE synthesis are discussed — from the erythropoietic system in man to the

erythropoietic and megakaryocytic systems in cow, guinea pig, horse, rabbit and rat, and almost exclusively to the megakaryocytic system in cat. On the basis of the available data the hypothesis is advanced that the erythropoietic and megakaryocytic systems derive from a common cell (chapter VII).

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